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A New *Azotobacter vinelandii* Mannuronan C-5-Epimerase Gene (*algG*) Is Part of an *alg* Gene Cluster Physically Organized in a Manner Similar to That in *Pseudomonas aeruginosa*

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Alginate is an unbranched polysaccharide composed of the two sugar residues β-D-mannuronic acid (M) and α-L-guluronic acid (G). The M/G ratio and sequence distribution in alginates vary and are of both biological and commercial significance. We have previously shown that a family of highly related mannuronan C-5-epimerase genes (*algE1* to *-E5*) controls these parameters in *Azotobacter vinelandii*, by catalyzing the Ca^{2+}-dependent conversion of M to G at the polymer level. In this report, we describe the cloning and expression of a new *A. vinelandii* epimerase gene (here designated *algG*), localized 29 nucleotides downstream of the previously described gene *algJ*. Sequence alignments show that *algG* does not belong to the same class of genes as *algE1* to *-E5* but that it shares 66% sequence identity with a previously described mannuronan C-5-epimerase gene (also designated *algG*) from *Pseudomonas aeruginosa*. *A. vinelandii algG* was expressed in *Escherichia coli*, and the enzyme was found to catalyze epimerization in the absence of Ca^{2+}, although the presence of the cation stimulated the activity moderately. Surprisingly, all activity was blocked by Zn^{2+}. *P. aeruginosa* AlgG has been reported to contain an N-terminal export signal sequence which is cleaved off during expression in *E. coli*. This does not happen with *A. vinelandii* AlgG, which appears to be produced at least partly in an insoluble form when expressed at high levels in *E. coli*. DNA sequencing analyses of the regions flanking *algG* suggest that the gene is localized in a cluster of genes putatively involved in alginate biosynthesis, and the organization of this cluster appears to be the same as previously described for *P. aeruginosa*.

Alginate is a polysaccharide produced by brown seaweeds and certain bacteria. In the seaweeds, the polymer probably serves an important function in determining the mechanical properties of the algal tissues. The combination of the availability of large quantities of seaweeds and the commercially interesting physical properties of alginates forms the basis for numerous applications of this polymer in industry and advanced biotechnology (25). The majority of these applications are based on the viscosity properties of alginate and on the ability of the polymer to form gels in the presence of divalent cations like Ca^{2+}.

Bacterial alginate synthesis has been identified and studied in organisms belonging to the genera *Pseudomonas* and *Azotobacter* (4, 10, 11, 15). Most of our knowledge on the genetics of alginate synthesis originates from studies of *Pseudomonas aeruginosa*, and these studies are mainly motivated by the problems that this pathogen causes for patients suffering from the disease cystic fibrosis. These problems strongly correlate with alginate production through the serious effects the polymer has on the respiratory system of the patients (16). In both *Azotobacter vinelandii* and *P. aeruginosa*, alginate is synthesized as an extracellular polysaccharide in vegetatively growing cells, but in *A. vinelandii*, the polysaccharide also plays a crucial role in the unique ability of this organism to enter a so-called cyst stage. In this state, the cells are protected from certain environmental stress conditions, like dehydration (20). *A. vinelandii* cysts are coated with an outer protective layer containing alginates of varying composition. This structural variation, which is also observed in different parts of individual seaweed plants, involves differences in the fractional ratios and distributions of the two sugar monomers β-D-mannuronic acid (M) and α-L-guluronic acid (G) along the unbranched polymer chains (13, 20). Bacterial alginates are in addition acetylated, while this is not the case for alginates from seaweeds (23).

It is well established that alginates containing blocks of G residues can form strong gels in the presence of divalent cations like Ca^{2+} (26). If the G residues are distributed in a more alternating sequence pattern, most of the gel-forming properties are lost (26). The sequence distribution of M and G residues is therefore important from both a biological and an application point of view. Alginates produced by *Pseudomonas* species do not contain G blocks, while such structures are found in brown seaweed and *A. vinelandii* alginates (13, 23). In two recent papers, we showed that the sequence distribution of M and G residues in *A. vinelandii* alginates is determined by the gene products (mannuronan C-5 epimerases) of a family of highly related genes (6, 7). The epimerases have the unusual property of converting M to G at the polymer level, and this reaction can take place in vitro in the absence of any cofactors other than the divalent cation Ca^{2+}. The existence of a mannuronan C-5 epimerase gene (*algG*) in *P. aeruginosa* has also been reported previously (9). Alignment analyses at the DNA and protein level showed that the *P. aeruginosa* gene (and its product) was quite different from its counterparts (*algE1* to *-E5*) in *A. vinelandii* (7).

The *P. aeruginosa algG* gene is localized within a cluster of genes encoding enzymes directly involved in different stages of the alginate biosynthetic pathway (16). Such genes were not found to be associated with the *A. vinelandii* epimerase genes. It has, on the other hand, been shown that probes prepared from *P. aeruginosa alg* genes hybridize against *A. vinelandii* genomic DNA (8). These data therefore indicated that there exists at least some level of similarity in the genes controlling

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TABLE 1. Bacterial strains and plasmids

Strain, phage, and plasmid	Characteristics	Reference or source
Strains		
E. coli JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*)	27
P. aeruginosa 8830	*his-1* Alg^+; stable mucoid mutant	5
Phage BR100	EMBL3A derivative containing a 17-kb insert from *A. vinelandii*; encodes *algJ*	18
Plasmids		
pQE60	Ap^r, ColE1 replicon	Qiagen
pUC19	Ap^r, ColE1 replicon	17
pUC128	Ap^r, ColE1 replicon	14
pBHR59	Derivative of pUC19 in which a 2.6-kb *Eco*RI fragment containing *A. vinelandii algG* was inserted	This paper
pBHR60	Derivative of pQE60 in which a 1.7-kb PCR-amplified *Nco*I-*Bgl*II fragment containing *algG* was inserted	This paper
pHE102	Derivative of pUC128 in which a 2.8-kb *Xho*I-*Sma*I DNA fragment from BR100 was inserted. Contains the 3′ end of the *A. vinelandii algG* gene and the two boxes having homology with *algX* and *algL* from *P. aeruginosa*	This paper
pHE104	Derivative of pHE102 in which a 1.1-kb *Eco*RV-*Xba*I (polylinker at *Sma*I end) DNA fragment was deleted	This paper
pHE105	Derivative of pUC19 in which a 3.3-kb *Eco*RI-*Sma*I DNA fragment from BR100 was inserted. Contains sequences homologous to the *P. aeruginosa alg8* and *alg44* genes	This paper
pHE106	Derivative of pHE105 in which a 1.0-kb *Eco*RI-*Nco*I DNA fragment was deleted	This paper
pHE107	Derivative of pUC19 in which a 6.0-kb *Sma*I-*Eco*RI DNA fragment from BR100 was inserted. Contains sequences homologous to *algD* and *alg8*	This paper
pHE108	Derivative of pHE107 in which a 5-kb region was deleted. The region spans the *Hin*dIII site in the vector polylinker (*Sma*I end of insert) to the *Xho*I site near box B in Fig. 5	This paper
pHE110	Derivative of pHE102 in which a 1.7-kb region from the *Xho*I to the *Eco*RV site was deleted. Contains sequences homologous to *algL* in Fig. 5	This paper

alginate biosynthesis in the two organisms. Recent experiments have verified this hypothesis directly by the identification of an *A. vinelandii* gene (*algJ*) encoding an outer membrane protein thought to be involved in alginate export (18). A similar gene (*algE*), encoding an outer membrane protein, has previously been reported in *P. aeruginosa* (2, 19). Furthermore, an *A. vinelandii* gene (designated *algD*) analogous to *P. aeruginosa algD* (encoding GDP-mannose dehydrogenase) was recently reported and was shown to be required for alginate synthesis in vivo in *A. vinelandii* (1). In *P. aeruginosa*, *algG* is localized next to *algE*, and we show here that a gene similar to *algG* is found in the same relative position in *A. vinelandii*. On the basis of these data and further DNA sequencing analyses, we conclude that the alginate biosynthetic genes may be similarly organized in the two organisms but that *A. vinelandii* (presumably in contrast to *P. aeruginosa*) has two systems for epimerization of M to G.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Strains and plasmids are listed in Table 1. *Escherichia coli* cells were grown at 37°C in L broth or on L agar (21) supplemented with ampicillin (100 μg/ml) when relevant. *P. aeruginosa* was grown in liquid medium A at 32°C, as previously described by Skjåk-Bræk et al. (23), or at 18°C on 3% agar medium containing (per liter) 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 1 g of glucose.

DNA manipulations and cloning of *algG*. DNA was sequenced according to the method of Sanger et al. (22). Plasmids used for determination of sequences homologous to the *P. aeruginosa alg* genes were pHE108 (*algD* homolog), pHE107 (*alg8* homolog, C1 [see Fig. 5]), pHE105 (*alg8* homolog, C2 [see Fig. 5]), pHE106 (*alg44* homolog), and pHE104-pHE110 (*algL* homolog). Vector primers were used in all these DNA sequencing procedures, while *algG* was sequenced by primer walking in both directions. The sequence homologous to *algX* was obtained from the data shown in Fig. 1. Standard recombinant DNA procedures were performed according to protocols described in the work of Sambrook et al. (21), except for transformations, in which the protocol of Chung et al. (3) was also used. Labelling of DNA for the Southern hybridization analysis was performed by the digoxigenin system from Boehringer Mannheim. The DNA fragment containing *algG* originated from the same recombinant EMBL3 phage (BR100) as *algJ* (18), and *algG* was identified by sequencing of the region downstream of *algJ*, with plasmid pBHR59 as template. High-level expression of *algG* was obtained by subcloning into the expression vector pQE60. The cloned fragment was prepared by PCR amplification with the synthetic oligonucleotides 5′ TCCAGCGGCACGCTGAGAGGATCGACCATGGA3′ (5′ end of *algG*) and 5′ CATTTGCTGTTGTAGAGATCTTCATGTT3′ (3′ end of *algG*). In this strategy, a *Nco*I site was introduced near the 5′ end and a *Bgl*II site was introduced near the 3′ end of the fragment. Both sites were used for cloning of the PCR fragment into the *Nco*I-*Bam*HI sites of the vector polylinker. The use of the *Nco*I site made it possible to position the first ATG optimally relative to the ribosome-binding site in the vector.

Preparation of [^{3}H]alginate. *P. aeruginosa* was first grown for 3 days in liquid medium, and 0.5 ml of the culture was then plated on each of six agar plates (diameter, 14.5 cm) and incubated for 24 h. [5-^{3}H]glucose (25 μCi per plate, 15.7 Ci/mmol; Amersham) was then added, and the plates were incubated further for 3 more days. The cells and exopolymer were scraped off the plates and resuspended in a total volume of 750 ml of 0.9% NaCl. The solution was then subjected to centrifugation to remove the cells, and the supernatant was filtered through a set of three membrane filters (1.2-, 0.7-, and 0.2-μm pore size, respectively). Deacetylation of the labelled alginate was performed by incubation for 1 h at room temperature after addition of NaOH to 0.1 M. The solution was then neutralized with HCl, dialyzed extensively against water, and then concentrated a factor of two by evaporation. NaCl was added to 0.2%, and the polymer was precipitated with an equal volume of 96% ethanol and washed extensively with 70% ethanol–chloroform–ether. After drying, the pellet was dissolved in water at 0.25%, filtered as described above, and finally freeze-dried. The composition of the labelled alginate was analyzed by ^{1}H nuclear magnetic resonance spectroscopy on a Bruker 500 WM spectrometer, as previously described (12).

Visualization of AlgG by SDS-PAGE and measurements of AlgG activity. Cells of strain JM109(pBHR60) were grown overnight in the presence of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and washed once in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) pH 7.4. Whole-cell lysates were prepared by resuspending the cells (10 times concentrated) in gel loading buffer. The cytosol fraction was prepared by disruption of the cells in a French press and by sedimenting cell debris with centrifugation (40,000 × *g*) for 1 h. The pellet (insoluble fraction) was resuspended in the above HEPES buffer. AlgG was identified as a protein band at 58 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in strains induced to express *algG* and not in vector (pQE60) control strains.

For measurements of AlgG activity, JM109(pBHR60) cells were inoculated at 1% from overnight cultures, and after 4.75 h, IPTG was added at 0.5 mM. Cells (900 ml) were harvested 2.5 h after IPTG induction, resuspended in 60 ml of 50 mM Tris–2 mM dithiothreitol–1 mM phenylmethylsulfonyl fluoride, and sonicated. After centrifugation at 30,000 × *g* for 30 min, the pellet was resuspended in 6 ml of 50 mM Tris–3% CHAPS {3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate}–0.5 mM NaCl (pH 7.0) and stored overnight without significant loss of activity. It was then centrifuged again at 30,000 × *g* for 30 min, and the supernatant was subjected to sterilization filtration. No activity was lost

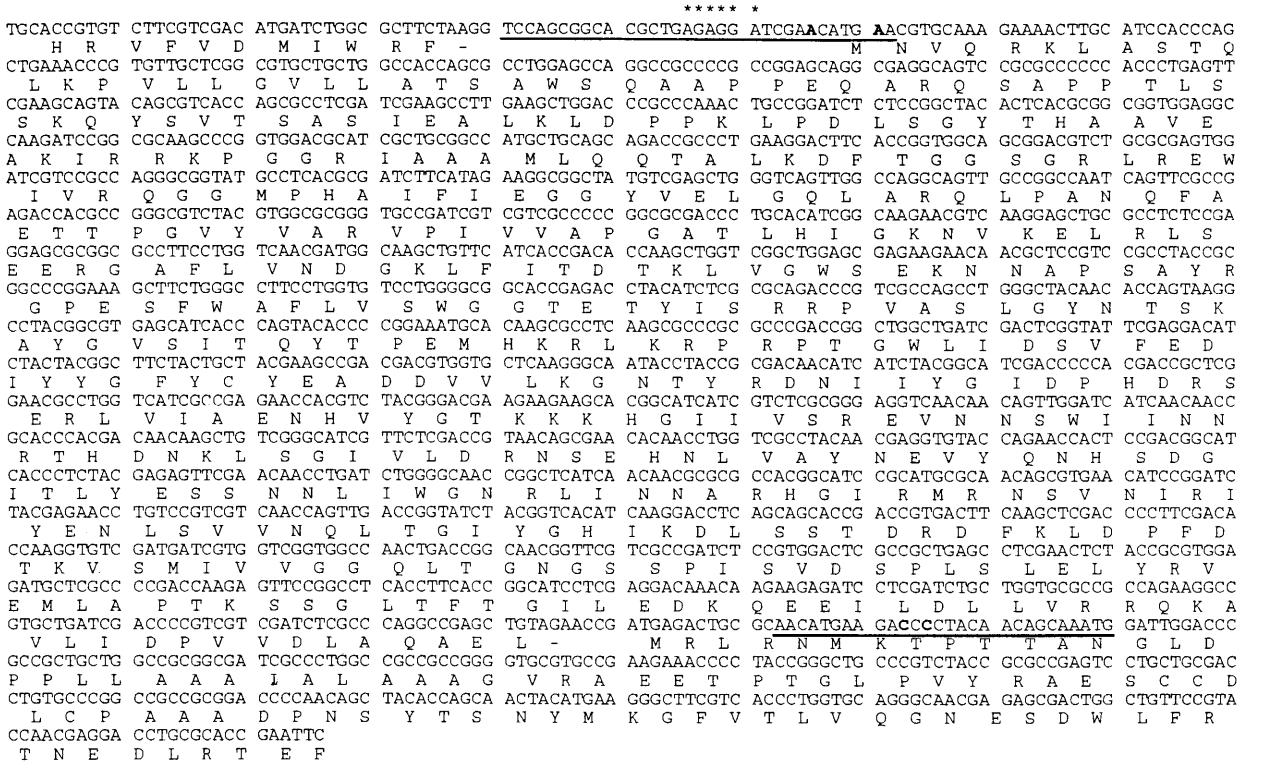

FIG. 1. Nucleotide sequence of *algG* and deduced amino acid sequence of its gene product. Bases in boldface were changed in the PCR cloning procedure (from 5′ end, A to C, A to G, C to T, and C to T). Note that this leads to an amino acid substitution from N to D at the second codon. A putative ribosome-binding site is indicated by stars. Sequences corresponding to the primers used for PCR cloning are underlined. The amino acid sequences upstream and downstream of AlgG represent the C terminus of *algJ* and the N terminus of the ORF having homology with the *P. aeruginosa algX* gene (Fig. 5).

in this last centrifugation and filtration step. The filtered solution was used for activity measurements.

Activity assays (see below) demonstrated that the AlgG activity in the cytosol fraction obtained directly after centrifugation was higher than that in the corresponding pellet, calculated as disintegrations per minute per original cell mass. However, by using the pellet for activity assays we obtained higher activities per weight unit of total protein. This turned out to be important, since the measured activities were low (see Table 2).

[^{3}H]alginate (84,000 dpm/mg) was used as substrate in the activity assays (24). The incubation mixtures (0.6-ml total volume) contained 0.25 mg of [^{3}H]alginate per ml, 0.1 ml of cell extract, and 30 mM Tris (pH 7.0), and divalent cations were added as indicated. The mixtures were incubated at 37°C for 12 h, and epimerase activities were monitored as released ^{3}H, as previously described (6) except that the alginate was precipitated with 0.8 ml of isopropanol.

N-terminal sequencing of AlgG. Aliquots of sonicated IPTG-induced cells from JM109(pBHR60) were loaded directly on an SDS-PAGE gel and subjected to amino-terminal sequencing, as previously described (6).

Computer analyses. The program PC/GENE (version 6.7) was used to determine the predicted isolectric point of *A. vinelandii* AlgG, while alignments were performed by the use of Seqaid.

Nucleotide sequence accession number. The *algG* nucleotide sequence data were deposited in the GenBank database under the accession number X87973.

RESULTS

Cloning and sequencing of a new mannuronan C-5-epimerase gene. The region downstream of the previously described *algJ* gene was subjected to DNA sequencing, and one long open reading frame (ORF) was identified (Fig. 1). A 1.5-kb DNA fragment containing this ORF was subcloned by PCR from the same recombinant λ phage (BR100) which was used for subcloning of *algJ*. This ORF is putatively transcribed in the same direction as *algJ*, and only 29 nucleotides separate the stop codon of *algJ* from the first codon in the ORF. The deduced amino acid sequence corresponds to a polypeptide with a molecular mass of 58 kDa (525 amino acids). A putative ribosome-binding site in the expected position was also identified. This arrangement is strikingly similar to *algE-algG* in *P. aeruginosa*, and the deduced amino acid sequence of the ORF identified here was therefore aligned against the *P. aeruginosa* AlgG sequence (Fig. 2). The analysis shows that the two sequences have 66% identity, indicating that the *A. vinelandii* ORF probably encodes an epimerase similar to that encoded by *algG* in *P. aeruginosa*. *P. aeruginosa* AlgG was previously shown to be quite basic, with a predicted isoelectric point of 8.9. A similar analysis of the putative polypeptide described here showed that the predicted isoelectric point is 9.4. On the basis of this and other experiments described below, the new gene in *A. vinelandii* was also assigned the designation *algG*.

Expression of the *A. vinelandii algG* gene. In the process of subcloning *algG*, a *Nco*I site was generated to facilitate insertion of the gene into the corresponding site of the ATG expression vector pQE60. The resulting plasmid, pBHR60, was used to express the polypeptide encoded by *algG*. Figure 3, lanes 1 and 2, shows that in the presence of IPTG induction a protein of 58 kDa was efficiently expressed in cells containing pBHR60 but is absent in cells containing the vector only. The protein was also visualized in the cytosol fraction (lanes 3 and 4) and in the pellet (insoluble fraction) obtained by centrifugation of the cells disrupted in a French press (lanes 5 and 6). This is in agreement with the results obtained in the activity assays (see below).

P. aeruginosa AlgG has been reported to contain an N-terminal export signal sequence which is cleaved off (35 amino acids) when the protein is expressed in *E. coli* (9). We have therefore also sequenced the N terminus of *A. vinelandii* AlgG expressed in *E. coli* and found that the sequence is X (uniden-

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A.v: MNVQRKL--------ASTQLKPVLLGVLLATSAWSQAAPPEQARQSAPPT-----------LSSKQYSVTSASIEALKLDPPKLP  66
P.a: MPDISLSIPRRRLPRLRPLAAAVLGAVLLHGQAW--AAQPVEKPQPVPAQAGNEPGLTQGLKETGNYTVTTAPAEPLHLDPPKLP  83

A.v: DLSGYTHAAVEAKIRRKPGGRIAA-AMLQQTALKDFTGGSGRLREWIVRQGGMPHAIFIEGGYVELGQLARQLPANQFAETTPGV 150
P.a: DLSGYTAAAVEAKIVRKPGGRASVQRMVQQQPLKEFTGGSNRLAEWVKRQRQMPQAIFIEGGYVNLAQLAGKLPASALEQVEPGV 168

A.v: YVARVPIVVAPGATLHIGKNVKELRLSEERGAFLVNDGKLFITDTKLVGWSEKNNAPSAYRGPESFWAFLVSWGGTETYISRRPV 235
P.a: FVARLPIVVSQGATLDIDKQVKELRLSQERGAFLVNDGMLFVRDSKVTGWSESKKEPAWFKTPNEFRPFLISWGGAEVYLSNSTF 253

A.v: ASLGYNTSKAYGVSITQYTPEMHKRLKRPRPTGWLIDSVFEDIYYGFYCYEADDVVLKGNTYRDNIIYGIDPHDRSERLVIAENH 320
P.a: TSFGYNASKAYGISISQYSPGMDKQMKRPRPKGWVIDSTIVDSWYGFYCYEADDLVVKGNTYRDNIVYGIDPHDRSHRLIIADNT 338

A.v: VYGTKKKHGIIVSREVNNSWIINNRTHDNKLSGIVLDRNSEHNLVAYNEVYQNHSDGITLYESSNNLIWGNRLINNARHGIRMRN 405
P.a: VHGTRKKHGIIVSREVNDSFIFNNRSYENKLSGIVLDRNSEGNLVAYNEVYRNHSDGITLYESGDNLLWGNQVLANRRHGIRVRN 423

A.v: SVNIRIYENLSVVNQLTGIYGHIKDLSSTDRDFKLDPFDTKVSMIVVGGQLTGNGSSPISVDSPLSLELYRVEMLAPTKSSGLTF 490
P.a: SVNIRLYENLAAGNQLIGVYGHIKDLTNTDRNIALDPFDTKVSLIVVGGKLAGNGSGPLSVDSPLSLELYRVAMLAPTKSSGISL 508

A.v: TGILEDKQEEILDLLVRRQKAVLIDPVVDLAQAEL                                                    525
P.a: PGVLGEKQDQILDLLVRQDKAVLIDPVESQAELQD                                                    543
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FIG. 2. Comparison of the AlgG sequences from *A. vinelandii* and *P. aeruginosa*. The *Pseudomonas* sequence was obtained from GenBank (accession number U06720). The export signal sequence in *P. aeruginosa* is underlined (9). A.v, *A. vinelandii*; P.a, *P. aeruginosa.*

tified)-V-Q-R-K. This sequence corresponds to the start of the deduced ORF shown in Fig. 1 and 2, and the data thus indicate that no signal peptide is cleaved off during production in *E. coli.*

Measurements of epimerase activity. Epimerase activity was assayed in extracts prepared from uninduced and IPTG-induced cells containing pBHR60. Under uninduced conditions, the activities were found to be low but detectable in this sensitive assay (not shown). The highest activity (100%) was observed in extracts from induced cells and with an assay buffer containing 5 mM $CaCl_2$ and 10 mM $MgCl_2$ (Table 2). In the absence of any of these metals, 39% of the activity was still retained. This remaining activity was also found to be essentially insensitive to the addition of 10 mM Na_2-EDTA, indicating that there is no absolute requirement for divalent metal ions. The properties of AlgG are therefore different from those of the strictly Ca^{2+}-dependent *A. vinelandii* epimerases (AlgE1 to -E5) previously described. Surprisingly, the addition of as little as 1 mM $ZnCl_2$ to the assay mixture completely abolished the activity, while $MnCl_2$ and Na_2MoO_4 had no significant effect (not shown).

The physical organization of many *alg* genes is similar in *P. aeruginosa* and *A. vinelandii*. On the basis of previously reported data (18) and the experiments reported here, it seems clear that *algJ* and *algG* correspond to and are organized in a manner similar to that of *algE-algG* in *P. aeruginosa*. Because of the previous identification of a complex system of epimerase genes in *A. vinelandii*, it was obviously of interest to know whether *algG* is a member of another multicopy gene family. To analyze this, we hybridized *A. vinelandii* genomic DNA against an *algG* probe, and the results of this analysis strongly indicate that there is only one copy of the gene in the genome (Fig. 4).

Since *algE* and *algG* are part of a cluster of *alg* genes in *P. aeruginosa*, it seemed probable that more *alg* genes might be localized in the *A. vinelandii* DNA sequences flanking *algJ-algG*. A restriction endonuclease map of the insert in BR100 was constructed (Fig. 5A), and several of the fragments flanking *algJ-algG* were then subjected to partial DNA sequencing. The sequences were translated into putative amino acid sequences in all three reading frames in both orientations and were then aligned with the previously reported *alg* gene sequences from *P. aeruginosa*. In each case, one of the reading

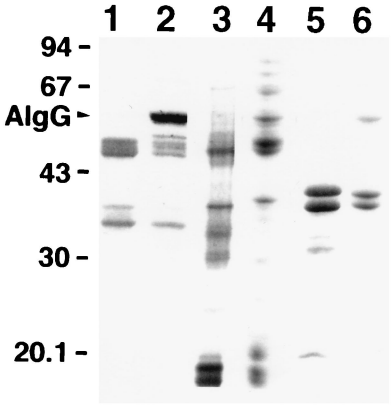

FIG. 3. Visualization of *algG* expression in *E. coli* by SDS-PAGE. Lanes 1, 3, and 5, whole-cell extracts, cytosol, and insoluble fraction (respectively) from JM109(pQE60). Lanes 2, 4, and 6, the same fractions from IPTG-induced JM109(pBHR60). The cytosol and insoluble fractions correspond to about 20% of the cells relative to the whole-cell extracts. The numbers to the left refer to a molecular mass standard (kilodaltons).

TABLE 2. Effects of divalent cations on AlgG activity (percent)[a]

Concn (mM) of salt	$CaCl_2$	$MgCl_2$	$ZnCl_2$	Na_2-EDTA
1	64	36	0	38
5	70	54	0	37
10	59	65	ND[b]	35
15	40	74	ND	ND

[a] One hundred percent activity was obtained in the presence of 5 mM $CaCl_2$–10 mM $MgCl_2$ and was measured as 2,020 dpm (blank subtracted). The blank value was 99 dpm. In the absence of added salts, the activity was 39%.

[b] ND, not determined.

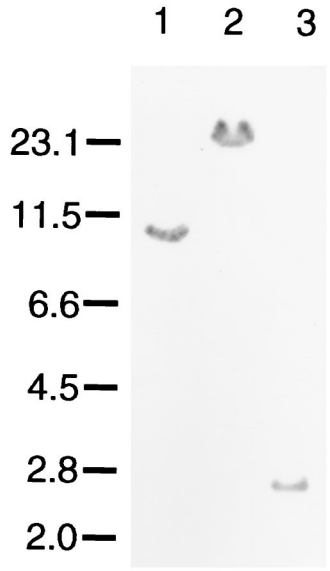

FIG. 4. Southern hybridization of *algG* against restriction endonuclease-digested chromosomal DNA from *A. vinelandii*. A 1.1-kb DNA fragment spanning the region from the *Hin*dIII site at nucleotide 710 (Fig. 1) to the polylinker *Hin*dIII site downstream of *algG* in pBHR60 was used as a probe. Lane 1, *Kpn*I digest. Lane 2, *Bgl*II digest. Lane 3, *Eco*RI digest. The numbers to the left refer to a molecular mass standard (kilobases). No signals were observed at lower molecular masses than those indicated.

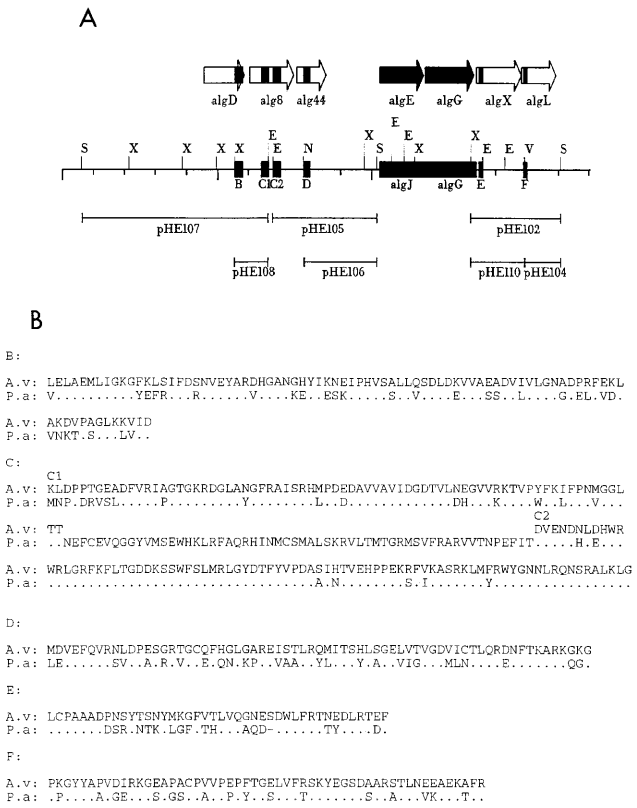

FIG. 5. (A) Restriction endonuclease map of the insert in the recombinant EMBL3 phage BR100. Black boxes on this map indicate DNA sequences used for alignments against *P. aeruginosa alg* genes. Each vertical bar in the map represents 1 kb. S, *Sma*I; X, *Xho*I; E, *Eco*RI; N, *Nco*I; V, *Eco*RV. There are many *Nco*I and *Eco*RV sites in the BR100 insert, and only those used for cloning are shown. The inserts in the plasmids used for the characterizations are indicated below the map. The corresponding map of the previously reported *P. aeruginosa* genes (16) is shown above the *A. vinelandii* restriction map in the same scale. Each black box in the *P. aeruginosa* map has homology with the corresponding box in the *A. vinelandii* map. (B) Sequence alignments between *P. aeruginosa alg* genes and *A. vinelandii* DNA flanking *algJ-algG*. The alignments are based on previously characterized *P. aeruginosa alg* genes and sequences from parts (B, C1, C2, D, E, and F) of the inserts in the plasmids shown in panel A. Note that the sequence 5′ to the black box (E) in the *A. vinelandii algX* homolog has also been determined (Fig. 1), but the deduced amino acid sequence did not have much homology with the *P. aeruginosa* AlgX protein. The same was true for the first nine deduced amino acids following immediately downstream of box D. With these two exceptions, all sequenced regions are shown, and the homology percentages in the aligned regions were calculated to be 64 (*algD*), 84 (*alg8*), 58 (*alg44*), 57 (*algX*), and 69 (*algL*). The *P. aeruginosa* sequences were obtained from GenBank and had the following accession numbers: Y00337 (*algD*), L22611 (*alg8* and *alg44*), M37181 (*algE*), L27829 (*algX* = *alg60*), and U09724 (*algL*). The *A. vinelandii algD* sequence reported by Campos et al. (1) is identical to the one shown here. The alignments of *algJ* and *algE* have been reported previously (18), and the *algG* comparisons are shown in Fig. 2. A.v, *A. vinelandii*; P.a, *P. aeruginosa.*

frames showed significant homology to specific parts of *alg* genes from this organism (Fig. 5B). Furthermore, the homologous sequences from *A. vinelandii* were all in the same orientation as in *P. aeruginosa*, and the relative positioning was the same. On the basis of these data, we conclude that the *A. vinelandii* genome encodes putative genes having extensive homology to the *P. aeruginosa algD*, *alg8*, *alg44*, *algE*, *algG*, *algX*, and *algL* genes, and they are all physically organized in the same way in the two organisms.

DISCUSSION

The structures of *P. aeruginosa* alginates are much simpler than those found in the complex polymer mixtures produced by *A. vinelandii*. It therefore is not too surprising to find that these differences are also reflected at the genetic level. The identification of a homolog of the *P. aeruginosa algG* gene in *A. vinelandii* was, on the other hand, unexpected by us, since it means that the genome of this organism encompasses two classes of epimerization genes. The biological significance of having two such systems for epimerization is not clear. However, alginate is produced by vegetatively growing *A. vinelandii* cells, and since the *algG* gene was found to be localized in a putative *alg* biosynthesis cluster (as in *P. aeruginosa*), it seems probable that *A. vinelandii algG* is expressed under vegetative growth conditions. The original cloning of the first *algE* gene (*algE2*) was based on a protein expressed in vegetatively growing cell cultures (5), and it therefore seems likely that both systems for epimerization are used by the cells under standard laboratory growth conditions. It is also clear that there are more sequences having homology with *algE1* to *-5* in the *A. vinelandii* genome (reference 7 and unpublished data), and until the complete set of genes involved in epimerization has been characterized, it will be difficult to obtain a full understanding of the role of these enzymes in the biology of *A. vinelandii*. Such studies will have to be performed under a variety of environmental conditions, since it is probable that some of the epimerase genes play a role in cyst formation under conditions of environmental stress.

The recombinantly produced AlgG protein from *A. vinelandii* appears to have a very low specific activity. On the basis of SDS-PAGE gels, expression levels are clearly very high, but in spite of this, we were unable to obtain a sufficiently high activity to study the epimerization pattern by nuclear magnetic resonance spectroscopy. We believe that the most probable explanation of this is that the majority of the protein is produced in an inactive or poorly active form. The reason for this is not clear but may possibly be related to the lack of process-

ing in *E. coli*, in contrast to what was observed with *P. aeruginosa* AlgG (9).

If the *algG* gene products behave similarly in the two species, it is likely that these enzymes cannot form G blocks, although this has not been shown directly, even in *P. aeruginosa*. Another interesting observation is that at least *A. vinelandii algG* does not seem to require Ca^{2+} for its activity, while this is an absolute necessity for the *algE* class of epimerases (6). It is tempting to assume that this difference is somehow taken advantage of by the organism, particularly since this cation is of crucial importance via its effects on alginate gel formation.

The finding that the genes putatively involved in other aspects of alginate biosynthesis are almost identically physically organized in *P. aeruginosa* and *A. vinelandii* demonstrates that the biosynthetic machinery has a common evolutionary origin in the two organisms. It therefore seems likely that the complex epimerization system apparently unique to *A. vinelandii* is a trait that evolved later primarily to allow formation of the resting stage characteristic of this organism. On the basis of the results reported here, it seems clear that it would now be interesting to perform functional cross-complementation experiments of genes between the two species, and the further studies of alginate biosynthesis in *A. vinelandii* should in general benefit from the accumulated knowledge of *P. aeruginosa.*

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